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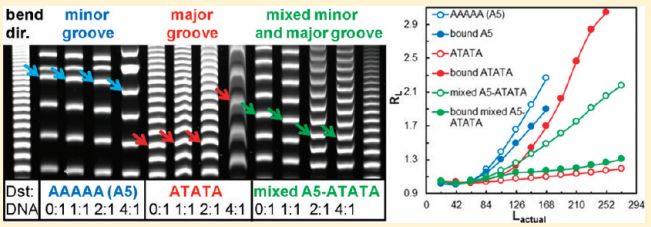
Minor Groove to Major Groove, an Unusual DNA Sequence-Dependent Change in Bend Directionality by a Distamycin Dimer

Shuo Wang, Manoj Munde, Siming Wang, and W. David Wilson*

Department of Chemistry, Georgia State University, Atlanta, Georgia 30303, United States

Supporting Information

ABSTRACT: DNA sequence-dependent conformational changes induced by the minor groove binder, distamycin, have been evaluated by polyacrylamide gel electrophoresis. The distamycin binding affinity, cooperativity, and stoichiometry with three target DNA sequences that have different sizes of alternating AT sites, ATAT, ATATA, and ATATAT, have been determined by mass spectrometry and surface plasmon resonance to help explain the conformational changes. The results show that distamycin binds strongly to and bends five or six AT base pair minor groove sites as a dimer with positive cooperativity, while it binds to ATAT as a weak, slightly anticooperative dimer. The bending direction was evaluated with an in phase A-tract reference sequence. Unlike other similar monomer minor groove binding compounds, such as netropsin, the distamycin dimer changes the directionality of the overall curvature away from the minor groove to the major groove. This distinct structural effect may allow designed distamycin derivatives to have selective therapeutic effects.



Natural and synthetic heterocyclic cations that bind to the DNA minor groove have demonstrated effectiveness as therapeutic agents as well as probes for use to extend our fundamental understanding of DNA molecular recognition.[1−14] Crystal structures with a variety of minor groove binding compounds have significantly extended our understanding of the structural varieties of these systems, the importance of solvent molecules in the complexes, and the induced fit effects for binding of both DNA and the bound small molecule.[1,15−22] There is significant recent progress in the development of minor groove-targeted therapeutics for cancer,[9,23,24] parasitic diseases,[25] and bacterial infections,[26,27] and this is an area of intense current research interest. Topics of specific importance in this research are the development of a greater variety of cell-permeable minor groove agents that have increased DNA binding sequence selectivity. Heterocyclic diamidines that target minor groove AT sequences in parasite mitochondrial kinetoplast DNA have shown excellent clinical activity in humans.[4,25] Polyamides based on the natural products netropsin and distamycin (Dst) have been developed with sequence recognition capabilities[2,11,28,29] much broader than those of most minor groove binders. The polyamides also have shown cell uptake potential, depending on the polyamide composition, cell type, and conditions.[30−32]

The successful sequence recognition development of the polyamides began with the discovery by Wemmer and co-workers[3,33,34] that distamycin could bind to some AT minor groove DNA sequences as a stacked dimer and to others as a monomer. Nuclear magnetic resonance (NMR)[35] and X-ray[36] structural studies of Dst−DNA complexes show that the polyamide can bind to four successive AT base pairs in the 1:1 mode. The binding mode, however, can change to a cooperative 2:1 stacked, antiparallel complex for some binding site sequences (for example, alternating AT base pair sequences) that contain five or six AT base pairs.[33,37] The extensive, repeated AT sequences in parasite kinetoplast DNA make such novel AT recognition an attractive development area for improved selectivity in parasite targeting.[7,25] As noted above, the extensive development of the Dst heterocyclic units to design compounds that can recognize a wide variety of different DNA base pair sequences and lengths, by Dervan, Sugiyama, Lee,[2,11,28,29] and co-workers, greatly increases the importance of this chemical group.

We have recently observed that many minor groove binding agents cause large conformational changes upon binding to alternating AT sequences, and such changes may be important for their mode of antiparasitic activity.[38,39] NMR data from the Wemmer group show that the dimer binding mode of Dst with ATATA has increased cooperativity over binding to AAAAA.[40] These results suggested that the cooperative dimer binding mode of Dst could induce significant changes in the local structure of AT sequences. Surprisingly, the effects of this well-studied and important minor groove binding polyamide on DNA structures have not been systematically evaluated. Such an evaluation along with the unique features of Dst binding as both a monomer and a dimer can help answer the question of

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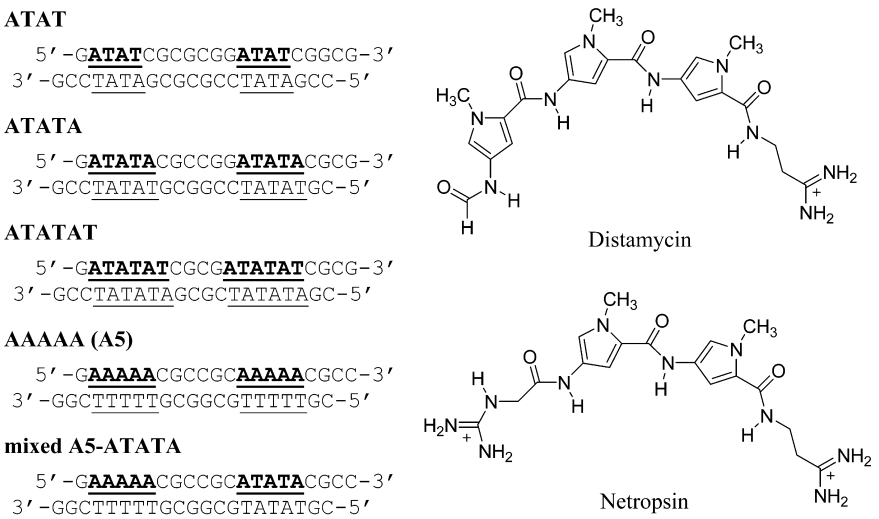

Figure 1. DNA sequences and compounds used in this study.

how the conformational changes induced by minor groove dimer complexes are distinct from those caused by monomers. This question has been addressed through several powerful methods: polyacrylamide gel electrophoresis (PAGE) with a ligation ladder assay, which is a very sensitive and well-established approach for detecting conformational changes in DNA that are induced by minor groove binders;[38,39,41,42] mass spectrometry of Dst complexes with alternating DNA sequences as a function of AT site length; and biosensor–surface plasmon methods. The results provide new insight into the Dst complexes at AT binding sites and show that the 2:1 cooperative binding of Dst induces significant bending effects in the DNA helix. The large bending by the Dst dimer is directed toward the major groove and differentiates Dst from minor groove monomer binders that typically cause bending of DNA sequences toward the minor groove.[38] This difference may allow Dst derivatives to have therapeutic mechanisms different from those of other similar minor groove binding drugs and provides important information for the development of specific inhibitors for transcription factors.

MATERIALS AND METHODS

DNA-Binding Compounds. Dst and netropsin were purchased from Sigma-Aldrich (St. Louis, MO), prepared as 1 mM stock solutions in doubly distilled water, and kept frozen at −4 °C in the dark. Purity was verified by NMR and mass spectrometry.

DNA Oligonucleotides. Three 21 bp duplexes with either 4, 5, or 6 bp alternating AT sites (ATAT, ATATA, or ATATAT, respectively, in Figure 1) and two 21 bp duplexes with two 5 bp AT sites with A tracts (AAAAA and mixed A5-ATATA in Figure 1) were used in this study. The standard marker, M21, is also a 21-mer sequence with the same base composition as the five AT base pair test duplexes but lacking AT binding sites.[38] An additional marker, a 20 bp ladder (Bayou Biolabs, Metairie, LA), is a commercially available standard sequence with a double intensity band at 100 bp for reference. Lyophilized DNA oligomers were purchased from Integrated DNA Technologies, Inc. (IDT, Coralville, IA) via HPLC purification. Doubly distilled water was added to the solid DNAs to bring the concentration to approximately 1.0 mM, based on the reported amount of DNA from IDT. The actual concentrations of these single-stranded DNAs were then determined using a Cary 300 UV–visible spectrophotometer (Varian, Walnut Creek, CA) at A_{260} with extinction coefficients calculated by the nearest-neighbor method.[43] Complementary strands were then combined in a 1:1 molar concentration ratio and annealed in ligation reaction buffer (New England Biolabs, Ipswich, MA) containing 50 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM dithiothreitol, and 1 mM ATP.

Ligation Ladders and Gel Electrophoresis. The ligation procedure is that described by Tevis[38] and Hunt.[39] Briefly, annealed duplexes were 5′-phosphorylated using T4 polynucleotide kinase (New England Biolabs) and then ligated with T4 DNA ligase (New England Biolabs) in the buffer provided. Kinasing reaction mixtures were incubated for 30 min at 37 °C followed by enzyme deactivation for 30 min at 65 °C. Ligation reaction mixtures in a volume of 200 μL contained 2 μM DNA with 1200 units of ligase, and reactions were conducted at room temperature for 20 min followed by enzyme deactivation for 30 min at 65 °C.

Ligation ladders were separated by 8% native polyacrylamide gels (1.5 mm thick, 20 cm long) prepared from a 40% acrylamide solution [29:1, bisacrylamide:acrylamide (Acros Organic)]. Electrophoresis was conducted at 200 V (10 V/cm) for 140 min at 25 °C in a Bio-Rad Protean II xi gel apparatus with TBE buffer [0.089 M Tris, 0.089 M boric acid, and 2.0 mM EDTA (pH 8.3)] and a Bio-Rad PowerPac Basic 300. Each sample had a final volume of 20 μL, which contained the following: 10 μL of 2 μM DNA, 4 μL of 6× load dye (Promega, Madison, WI), the amount of compounds needed for the desired ratios of compound to binding site, and doubly distilled water to reach the final volume. After electrophoresis, gels were stained with SYBR Gold Nucleic Acid Gel Stain (Invitrogen, Carlsbad, CA) at the concentration and time recommended by the manufacturer. The stained gels were imaged using an UltraLum Omega 10 gD Molecular Imaging System (UltraLum, Claremont, CA).

Calculation of R_L. Data for migration analysis and molecular weight assignments for ligation ladder products were obtained using Image Quant TL (Amersham Biosciences, Piscataway, NJ) and were based on the mobility of the ligated M21 duplex. The relative mobility R_L ($R_L = L_{apparent}/L_{actual}$[38,44]) was calculated for each ligated multimer. The mobility of each

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band of standard M21 was determined and correlated with its length in base pairs (L_{actual}) and molecular weight. The mobilities of each band of the target DNAs and the DNA−Dst complexes were determined and correlated to apparent base pair lengths ($L_{apparent}$) and molecular weights based on the standard values.

Mass Spectrometry. Mass spectrometry (MS) experiments were conducted with a Waters (Milford, MA) Micromass Q-TOF micro (ESI-Q-TOF) instrument operating in negative mode. The capillary voltage was set to 2200 V, the sample cone voltage to 30 V, and the extraction cone voltage to 3 V. The source block temperature and the desolvation temperature were set to 70 and 100 °C, respectively. Because mass spectrometry generally requires DNA sequences dissolved in a volatile salt solution, all the sequences tested with mass spectrometry were desalted three times by dialysis with a 1000 Da cutoff membrane (Spectrum Laboratories Inc., Rancho Dominguez, CA) in 0.1 M ammonium acetate (NH_4OAc, Fluka, Sigma-Aldrich) buffer to remove other salt ions from DNA, and the actual concentrations of the dialyzed DNA were determined as described above. Samples containing 5 μM target DNA sequences and appropriate amounts of Dst at the desired molar ratios with respect to DNA were prepared in 0.1 M NH_4OAc buffer with a final volume of 100 μL. The sample was introduced into the ion source by direct injection at a flow rate of 5 μL/min. DNA and Dst−DNA complexes were examined in the negative ion mode of the electrospray ionization (ESI) mass spectrometer, resulting in deprotonation and anion formation as shown in eq 1.

$$\text{gas phase ionization: DNA-}n\text{H}^+ \rightarrow [\text{DNA-}n\text{H}]^{n-} \quad (1)$$

where n is the number of protons removed from DNA to give the charge state. After the deprotonation, the multiply charged DNAs are evaluated according to the mass (m) to charge (z) ratios. MassLynx version 4.1 was used for processing the data.

Biosensor Surface Plasmon Resonance (SPR). SPR measurements were performed with a four-channel Biacore 2000 optical biosensor system (Biacore Inc.) as previously described. Three 5′-biotin-labeled DNA hairpins that contain the target alternating AT binding sites with the loop sequence underlined (5′-GGATATATCGTCTCCGATATATCC-3′, 5′-GGATATACGTCTCCGTATATCC-3′, and 5′-CGATATCGTCTCCGATATCG-3′) were immobilized onto streptavidin-coated sensor chips as previously described.[45−47] The experiments were conducted in a cacodylic acid buffer (6.25 pH, 100 mM NaCl and 0.005% P20 surfactant). In a typical experiment, 200 μL of samples at different concentrations were injected onto the chip surface at a flow rate of 25 μL/min and a 600 s dissociation period. The surface was regenerated with a pH 2.5 glycine solution followed by multiple buffer injections. Steady-state analyses were conducted, and data were fitted with either a two-site model or a one-site model ($K_2 = 0$):

$$r = (K_1C_f + 2K_1K_2C_f^2)/(1 + K_1C_f + 2K_1K_2C_f^2) \quad (2)$$

where r represents the moles of bound compound per mole of DNA hairpin duplex, K_1 and K_2 are macroscopic binding constants, and C_f is the free compound concentration in equilibrium with the complex. C_f is fixed by the concentration of the compound in the injected solution and is thus constant because the solution is continuously replaced in the flow cell. Binding thus occurs at constant C_f, while the free DNA and complex concentrations change. The observed steady-state response, RU_{sat}, at saturation of binding sites divided by the calculated response per bound compound, RU_{cal}, gives the maximal binding stoichiometry (n) of the DNA−compound complex, as shown in eq 3:[47]

$$n = RU_{sat}/RU_{cal} \quad (3)$$

RESULTS

ESI Mass Spectrometry: Binding Stoichiometry Analysis. ESI mass spectrometry analysis is a well-established method for the study of nucleic acid complexes.[48,49] On the basis of the mass:charge ratios and the relative peak intensities for binding of Dst with DNA duplexes containing alternating AT binding sites of 4−6 bp [ATAT, ATATA, and ATATAT (Figure 1)], the same DNAs used in the ligation experiments, the binding stoichiometry and cooperativity of Dst can be determined. The ESI mass spectra for free DNAs and Dst−DNA complexes were detected at five charge states ($-z$ = 10, 9, 8, 7, and 6), with the −7 and −8 peaks being dominant (Figure 2 and Figures S1−S3 of the Supporting Information). Peaks labeled with [1:2], [2:2], [3:2], and [4:2] in the figures represent the ratios of Dst ligands to the two binding sites in the duplex DNAs. The [2:2] and [4:2] labels stand for the 1:1 and 2:1 Dst−DNA binding sites in the complexes, respectively. The [1:2] and [3:2] ratios correspond to complexes having the two binding sites occupied by one and three (1:1 and 2:1 complexes) Dst molecules, respectively. When ATAT was treated with Dst at a 1:1 compound:DNA binding site ratio, the most abundant peaks observed were from the 1:1 complexes, whereas for ATATA and ATATAT under the same conditions, 2:1 complexes have increased intensity. In the mass spectra of the samples prepared with a 2:1 Dst:DNA binding site ratio, the 2:1 complexes became the most abundant species in all three tested duplexes and were clearly dominant in ATATA and ATATAT complexes. A hairpin DNA with an ATATA site, the same sequence tested in SPR but without the biotin label, was also treated with Dst under the same conditions. The 2:1 complex was the predominant species (Figure S4 of the Supporting Information), which is in agreement with the observation of the corresponding duplex sequence. The similar observations with duplex and hairpin sequences validate the use of hairpin sequences in our SPR studies. We have also made similar observations with duplex and hairpin sequences in ITC and SPR experiments.[50]

Surface Plasmon Resonance: Determining Affinity, Cooperativity, and Stoichiometry. Panels A−C of Figure 3 show biosensor SPR sensorgrams of Dst binding to ATAT, ATATA, and ATATAT hairpin DNA sequences, respectively. The biosensor SPR method provides detailed information about the affinity, stoichiometry, and cooperativity of DNA interactions.[45] As the concentrations of Dst are increased from 1 nM to 1 μM, the number of response units (RU) increases until around 80 nM for ATATAT and ATATA (Figure 3A,B), but significantly larger amounts are required to reach saturation with ATAT (Figure 3C). To evaluate the affinity and cooperativity for the interaction of Dst with the different AT sequences, the RU values at each concentration were determined in the steady-state region, where the on and off rates are equal and there is no mass transfer interference, and are plotted versus the C_f values for Dst, the concentration of the compound in each flow solution (Figure 3G). The fitting results in Figure 3G provide several key pieces of information

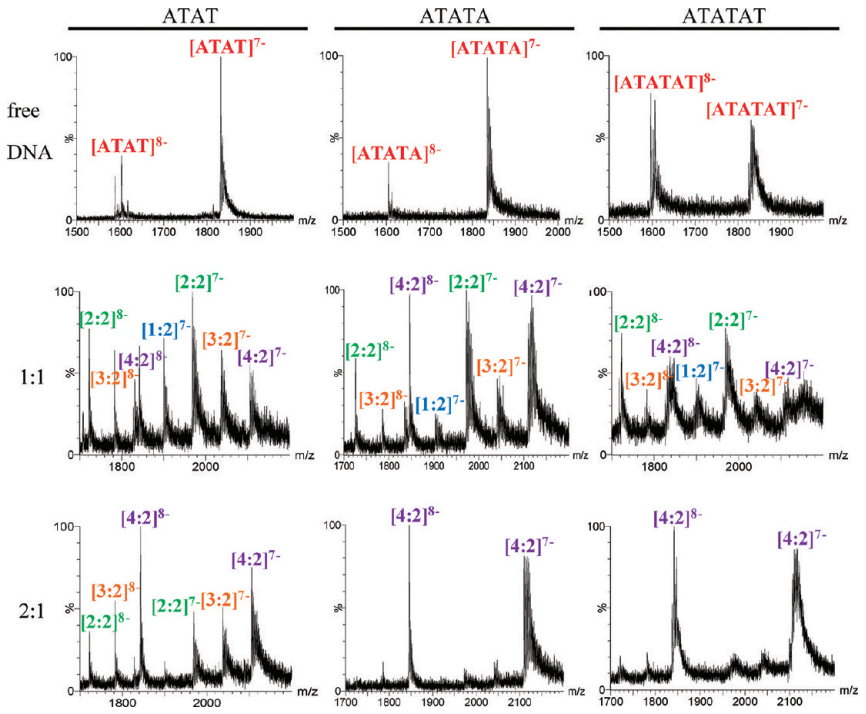

Figure 2. ESI mass spectra of Dst with the three target DNA duplexes containing two alternating AT sites at two compound:binding site concentration ratios (1:1 and 2:1). The label [*n*:2] (*n* = 1, 2, 3, or 4) represents the two binding sites that have been bound by *n* ligands of Dst at two charge states (−*z* = 8 and 7).

about the interaction of Dst with different DNAs. First, the predicted value per bound compound, based on the amount of immobilized DNA on the chip, is approximately 30 RU, and the observed value at saturation was between 55 and 60 RU, indicating that two molecules bind at the AT sites at saturation for all three DNAs. The sigmoidal behavior of the binding curves for ATATAT and ATATA indicates that Dst binds as a strong dimer with positive cooperativity (cooperativity factors of 40 for ATATAT and 28 for ATATA[51]). For ATAT, the SPR curve is hyperbolic and indicates noncooperative dimer formation at this site with an affinity lower than those for the longer sites. The binding constants for Dst binding to all three DNAs are listed in Table 1. For a clearer comparison of the binding affinity with target sequences, the average binding constant *K* on a per molecule basis of bound Dst, the square root of the product K_1K_2 [$(K_1K_2)^{1/2} = K$], is also reported in Table 1.

Netropsin is a well-known minor groove binder that has been thought to bind as a monomer regardless of the number of AT bases in the binding site.[1,3,8] The netropsin binding profile was compared to those with Dst with the same DNAs and shows a very different pattern. The observed RU values for netropsin at saturation (Figure 3D−F) with AT sequences are approximately half of those with Dst (Figure 3A−C), indicating that, as expected, netropsin recognizes these sequences as a monomer as opposed to the Dst dimers. At low concentrations, netropsin binds with slow kinetics for all DNA sequences, and it is not possible to reach a steady-state plateau in the experimental time range, which is limited by the injection volume. Even for these low-concentration samples, it is possible to fit the curves with a 1:1 binding model to determine predicted steady-state RU values for netropsin for use in Figure 3H. At the higher concentrations of netropsin, the steady state was reached, and these values can be used directly in the RU versus C_f plots along with predicted values. The binding constants determined in this manner are listed in Table 1. Netropsin binds with very high *K* values in the following order: ATATAT > ATATA > ATAT. Comparison of panels A−F of Figure 3 clearly shows that the DNA sequences with five and six AT base pair sites with Dst and all three DNAs with netropsin have relatively slow on and off rates, but the rates are much faster with ATAT and Dst.

Gel Electrophoresis: Binding-Induced Bending and Directionality. Ligation ladders of the three alternating AT target DNA oligomers without and with Dst are compared using PAGE, as shown in Figure 4A. Each oligonucleotide contains a two-base overhang to facilitate cohesive end ligation. Dst was incubated with ligated sequences at three concentrations to give 1:1, 2:1, and 4:1 compound:binding site ratios. In Figure 4A, as indicated by the colored arrows, the 189 bp ligated, linear multimers were used to compare the electrophoretic mobilities of each sequence. They were chosen because the free DNAs and complexes of this size have migrated long enough in the gel to be fully separated from the other ligated multimers and can display conformational features more pronounced than those of shorter or longer multimers under these conditions. The gel results show that the alternating AT duplexes are relatively straight because their ligated multimers migrated in the gel at the approximate rates of the reference sequence, M21, consistent with the findings of Tevis.[38] After binding with Dst, ATAT did not show significant mobility changes at any ratios. In contrast, an extremely pronounced retardation in migration could be observed for ATATA and ATATAT with an increase in the added concentration ratio to 4:1. However, as shown in Figure 4B, the extent of retardation did not change when the amount of compound was further increased, giving a final ratio of 10:1. In Figure 4C, the relative mobility, R_L, is plotted as a function of L_{actual} to compare the mobility changes induced by drug binding. The significantly

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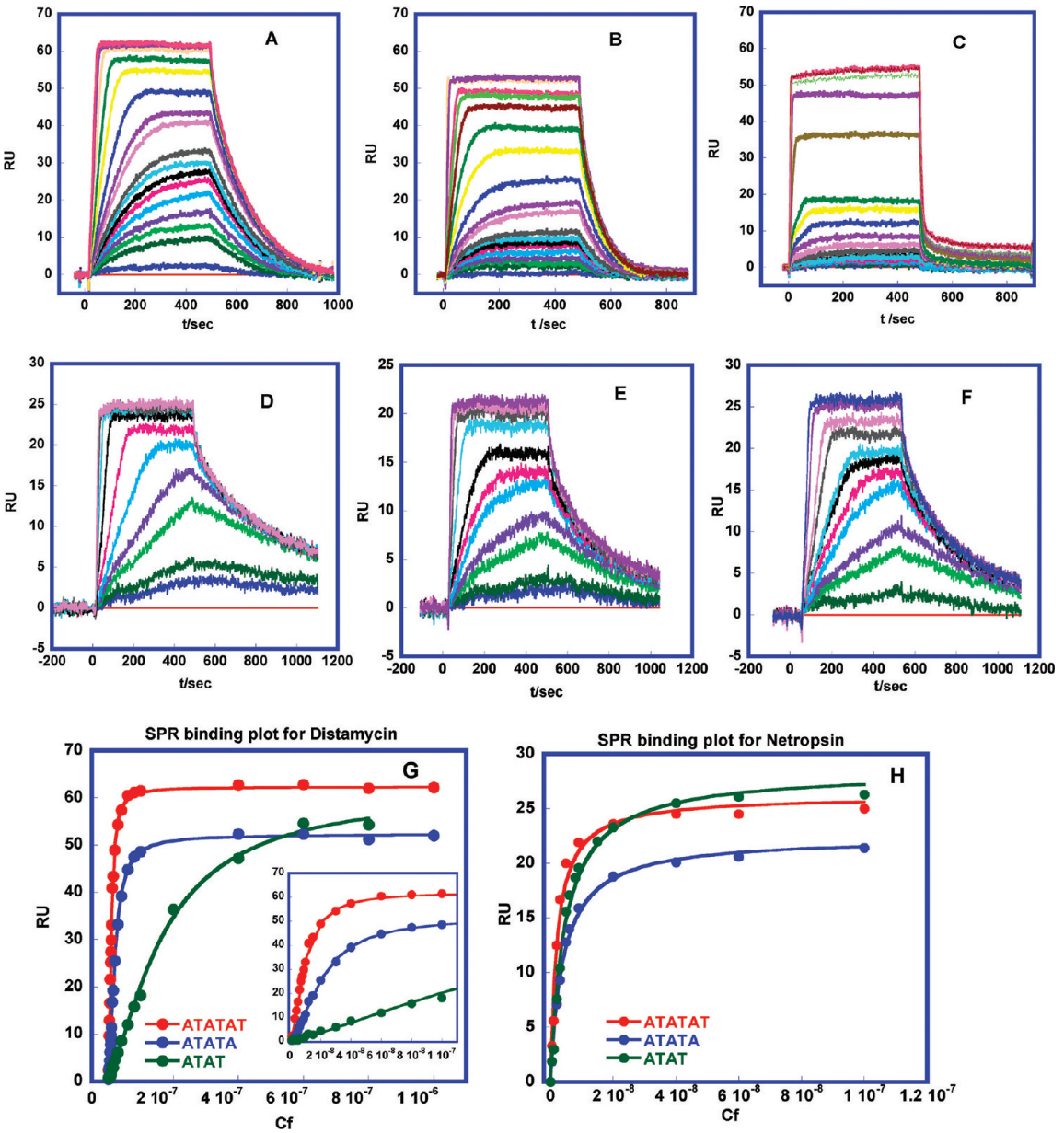

Figure 3. (A–C) Biosensor SPR sensorgrams (instrument responses in RU values vs time) for binding of Dst to (A) ATATAT, (B) ATATA, and (C) ATAT at 25 °C. The unbound compound concentrations in the flow solutions range from 0.001 μM in the bottom curve to 1 μM in the top curve. Experiments were conducted in cacodylic acid buffer with 0.1 M NaCl. (D–F) Biosensor SPR sensorgrams for binding of netropsin to (D) ATATAT, (E) ATATA, and (F) ATAT. The unbound compound concentrations in the flow solutions range from 0.001 μM in the bottom curve to 0.1 μM in the top curve. (G and H) SPR binding plots for (G) Dst and (H) netropsin in the presence of ATATAT, ATATA, and ATAT. The RU values from the steady-state region of the SPR sensorgrams are plotted vs the unbound compound concentration.

Table 1. Biacore Equilibrium Binding Constants[a] for Dst and Netropsin

	ATATAT	ATATA	ATAT
Dst	K_1 = 3.0; K_2 = 38	K_1 = 1.7; K_2 = 13	K_1 = 0.8; K_2 = 0.3
	K = 10.7	K = 4.7	K = 0.5
netropsin	46	24	21

[a]All K values have been multiplied by 10^{-7} and must be multiplied by 10^7 (M^{-1}) to obtain the values.

reduced mobilities and increasing R_L values indicate the DNA sequences containing five or six successive alternating AT bases were remarkably bent upon formation of the dimer by Dst in the longer sites.

It has been found that consecutive adenine, A-tract, sequences bend DNA in the direction of the minor groove.[38,52] A mixed sequence containing one AAAAA and one ATATA tract in phase was ligated and used to determine the directionality of Dst-induced ATATA bending with AAAAA as an internal bending direction reference. As shown in Figure 5A, the 168 bp, instead of the 189 bp (Figure 4A), ligated linear DNAs were used to compare the electrophoretic mobilities of each sequence, because the intrinsically curved AAAAA formed minicircles with the 189 bp multimers. The corresponding relative mobility of each target oligomer was calculated on the basis of the size in base pairs (Figure 5B). Dst induced opposing mobility changes in AAAAA and ATATA, which can be seen via comparison of the mobilities of the 4:1 ratios.

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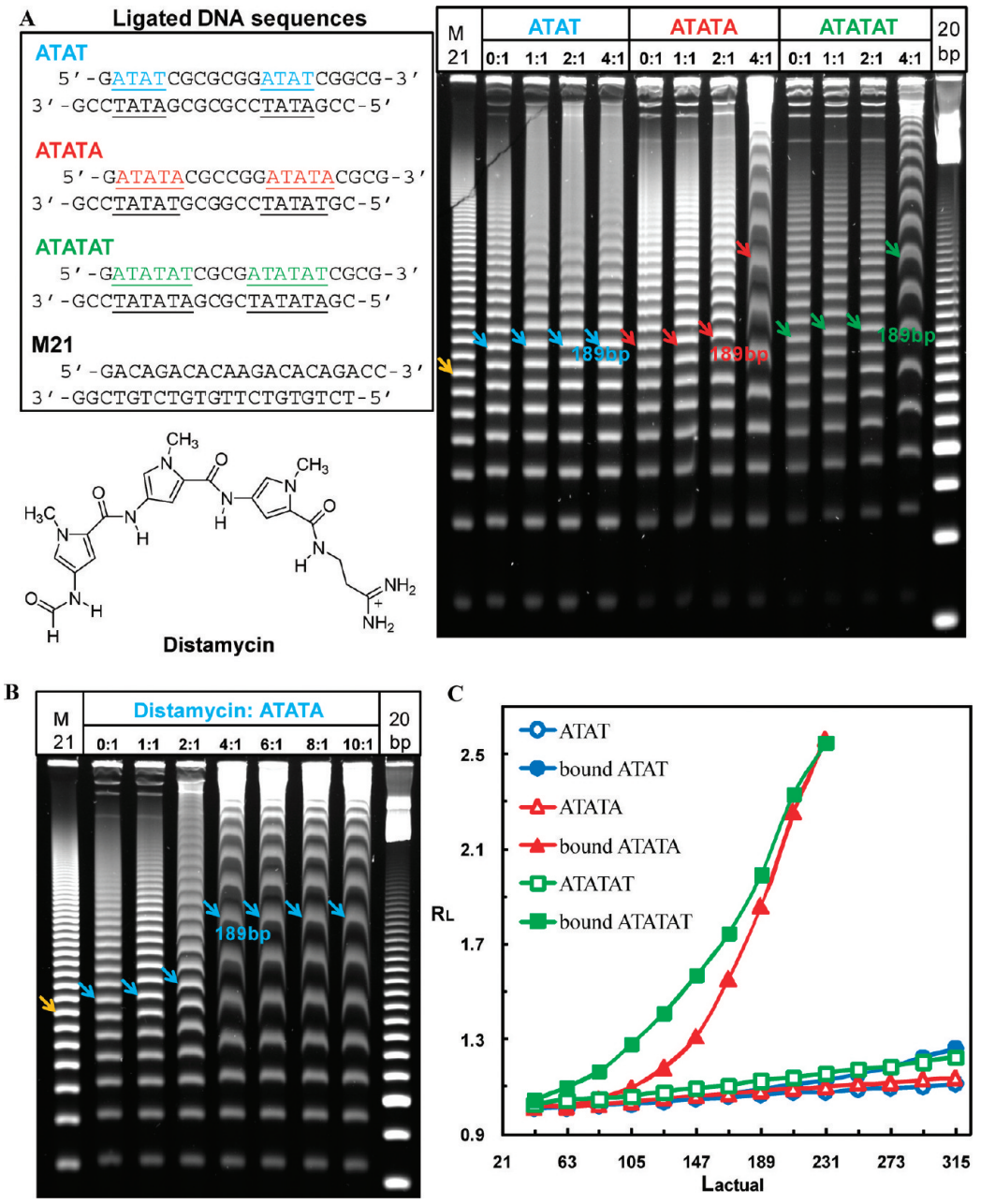

Figure 4. (A) 8% (29:1) native PAGE gel of Dst with three target DNA sequences: ATAT, ATATA, and ATATAT. Ratios atop the gel refer to compound:DNA binding site ratios. A 21 bp marker (M21) and a 20 bp marker are shown as the migration standards. Colored arrows demonstrate migration changes of target sequences and indicate 189 bp ligated linear multimers. (B) 8% (29:1) PAGE gel of Dst with ATATA at seven concentration ratios. Colored arrows indicate 189 bp multimers. (C) Plot of the relative mobility, R_L, as a function of L_{actual} for the three ligated duplexes in the absence (empty symbols) and presence (filled symbols) of Dst in a 4:1 compound:DNA molar ratio.

The slight increase for AAAAA and a remarkable reduction for ATATA in migration result in similar migration rates for these two Dst complexes. This similarity in mobility illustrates that the curvatures of bound AAAAA and ATATA are approximately the same. If both sequences are bent in the same direction, then the observed curvature for the bound mixed sequence will be approximately equal to that of bound AAAAA or ATATA. However, if ATATA bends in a direction opposite that of AAAAA, the bending effects will be partially canceled out and will result in a straighter overall structure similar to that of free ATATA. In a striking result in the presence of Dst, the bound mixed sequence in Figure 5A migrated much faster than the free one, and at a rate similar to that of free ATATA. This much less curved overall structure illustrates that the direction of the bend of ATATA induced by dimer binding of Dst is opposite that of AAAAA.

DISCUSSION

Dst is a very important minor groove binding agent because it was the first compound to have experimental proof for cooperative, stacked dimer formation in ≥5 bp binding sites of DNA.[33,34,37,40] It is also one of the few minor groove binding compounds to show dimer formation in pure AT sites. To improve our understanding of the Dst dimer formation in AT sites, and its effects on DNA structure, an important step in developing additional AT specific dimers with cell uptake as

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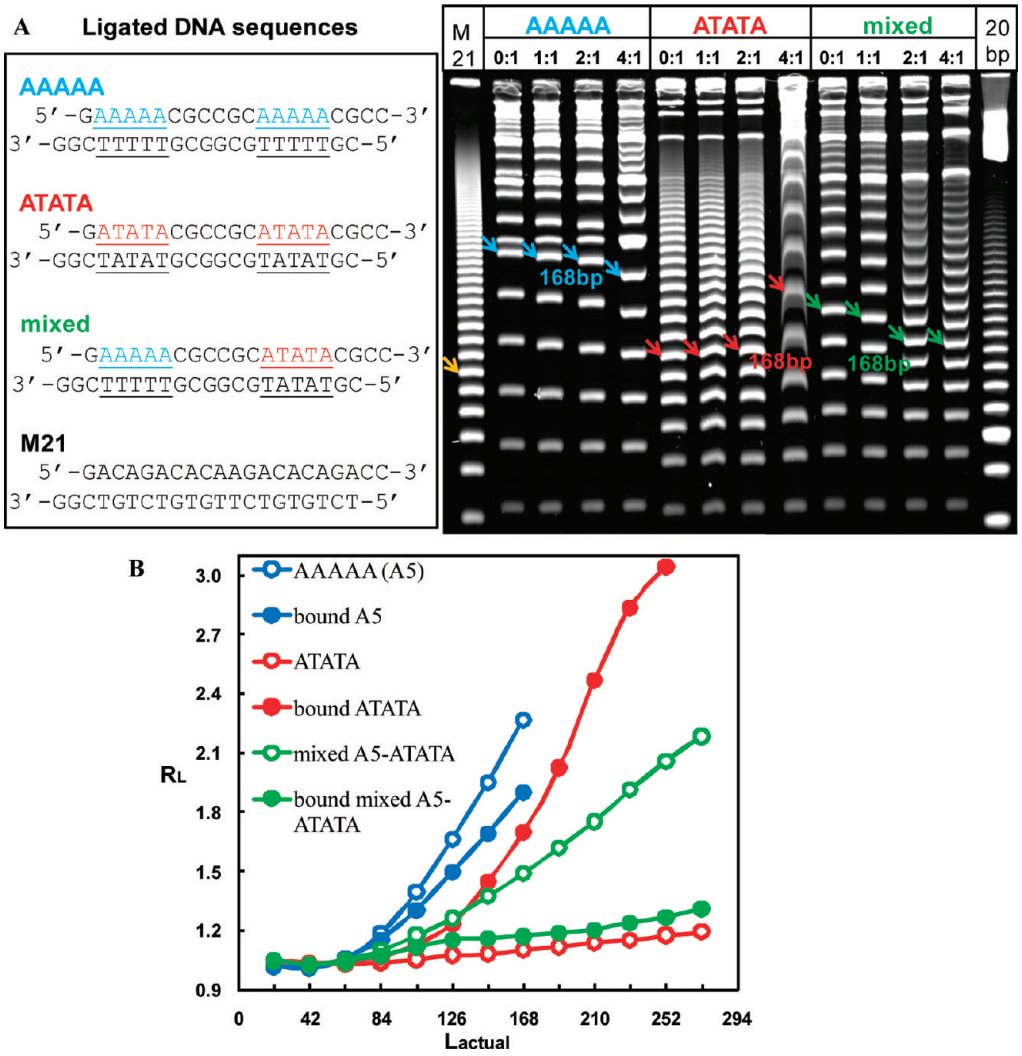

Figure 5. (A) 8% (29:1) native PAGE gel of Dst with three target DNA sequences: AAAAA, ATATA, and mixed A5-ATATA. Ratios atop the gel refer to compound:DNA binding site ratios. M21 and a 20 bp marker are shown as the migration standards. Colored arrows indicate 168 bp ligated linear multimers. (B) Plot of R_L as a function of L_{actual} as in Figure 4.

therapeutic agents, we evaluated conformational changes induced by Dst binding to AT sequences by using PAGE ligation ladder assays, ESI mass spectrometry of complexes, and SPR binding determinations with ATAT, ATATA, and ATATAT. These sequences were selected on the basis of the ground-breaking NMR studies that suggested a 1:1 Dst complex is preferred in ATAT, while the 5 and 6 bp sites can form a 2:1 stacked dimer.[33,40] The alternating AT sequences possess a wider minor groove than A-tract sites, and this significantly changes their affinity for 1:1 and 2:1 Dst complexes relative to A-tract DNA binding sites.[40]

In the ESI mass spectra of Dst with ATATA and ATATAT, at a 2:1 ratio, the dimer complex is the only one observed (Figure 2), while Dst preferentially forms a monomer complex and a weaker dimer at ATAT sites. A more quantitative picture of formation of Dst complexes with these sequences was determined with biosensor SPR technology. Dst shows very interesting differences in its binding profile in SPR depending on the number of AT base pairs in the binding site. A strong dimer with positive cooperativity is observed with ATATAT and ATATA, while a weaker and noncooperative dimer forms in ATAT. This is in agreement with mass spectrometry results that show that the monomer binding of Dst to ATAT is detected as the dominant species when the compound is present below molar concentrations relative to the DNA sequence, while a 2:1 complex becomes the most abundant species at high Dst:ATAT molar ratios. The binding features observed via mass spectrometry and SPR of Dst with selected target DNA sequences help with our understanding of the different conformational changes observed via PAGE.

The gel electrophoresis method used here has been validated as a qualitative tool for analyzing DNA conformational changes induced by minor groove binders.[39] To overcome partial dissociation of complexes in the gel experiment, high Dst:DNA binding site molar ratios (Figure 4B) were evaluated, and the complexes that formed at ratios above 4:1 displayed an identical electrophoretic mobility. This observation shows that once the binding sites are saturated to form the dimer, the DNA structural change is maximized and will not be further affected by added compound. Therefore, a ratio of compound to DNA higher than the binding stoichiometry is necessary to surmount the influence from dissociation of the compound in PAGE and to evaluate the full changes induced by the bound compounds.

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The molar ratio of 4:1 was consequently used in the calculation of relative mobility R_L.

The PAGE results and R_L values in Figures 4 and 5 show that at lower ratios (1:1 and 2:1) of Dst added per binding site, monomer binding of Dst had little effect on DNA conformation, while at higher ratios (≥4:1) a Dst dimer induced a cooperative change and significantly bent ATATA and ATATAT. In contrast, Dst does not have a detectable effect in the gel on the DNA sequence containing ATAT. As detected by SPR (Table 1), the second binding constant of Dst with ATAT is significantly less than 1×10^7 M^{-1}. A K value in this range indicates that the binding is not strong enough to ensure dimer formation in the gel experiment.[39] During the process of electrophoresis, weakly bound ($K < \sim 5 \times 10^6$ M^{-1}) compounds can dissociate from the negatively charged DNA sequence and, if they have a positive charge, migrate toward the opposite electrode. Consequently, only the monomer binding of Dst reaches a significant amount for ATAT, and little change in the ATAT structure can be detected by PAGE. Interestingly, the ATATA sequence is strongly bent by the monomer binding of netropsin and many heterocyclic diamidines,[38,39] while the Dst monomer with ATATA (1:1 and 2:1 ratios) shows only small conformational effects. The same effects are observed with the ATATAT binding sites at lower ratios and at all ratios with ATAT. The lack of an effect of Dst monomer binding is opposite to that with netropsin, which causes a marked bending of ATATA.[38] The lack of bending cannot be due solely to the fact that Dst is a monomer because other monomers cause large changes in ATATA structure.[39] Dst does bind weakly as a monomer to all alternating AT sequences, and it apparently is not able to compress the minor groove of AT sequences in a monomer complex, as with other minor groove binders.

To evaluate the directionality of curvature of the ATATA and the bend induced by Dst dimers, a mixed sequence that contains an AAAAA site as a curvature reference that bends DNA toward the minor groove and an ATATA test binding site in phase was designed. This kind of mixed sequence containing a target binding site as well as a reference site has been verified as a useful tool in studies of induced DNA bend directionality by covalent[10] and noncovalent minor groove binders.[38] In contrast to the results described by Tevis[38] with a number of minor groove binding agents, the relatively fast migration rate and the increased R_L value of the Dst-bound mixed sequence (Figure 5) reveal that Dst-induced bending is directed toward the major groove. Because the bending angles of free AAAAA and ATATA are 18° and 5°, respectively,[38] and the bound mixed sequence migrates at a similar rate with free ATATA, we estimate the bending angle of bound ATATA with Dst dimer is 15−20°. This result shows that the strong dimeric binding of Dst to ATATA not only remarkably bends this slightly curved sequence but also changes the directionality of the overall curvature away from the minor groove, the bend direction observed for monomer minor groove binders, to the major groove. This distinctive conformational effect might allow Dst or other polyamides (refs 20 and 21, for example) to act as an allosteric modulator of DNA transcription by altering the shape of the major groove and thus reducing the binding affinity of certain DNA transcription factors. This mechanism is different from the suggested mode of action for current minor groove binding antiparasitic drugs.[7]

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

ESI mass spectra of Dst with target duplex DNA sequences at five charge states and with a hairpin sequence containing an ATATA site (Figures S1−S4). This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Telephone: (404) 413-5503. Fax: (404) 413-5505. E-mail: wdw@gsu.edu.

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■ ABBREVIATIONS

Dst, distamycin; PAGE, polyacrylamide gel electrophoresis; ESI, electrospray ionization; MS, mass spectrometry; SPR, surface plasmon resonance; RU, response units of the SPR instrument; ITC, isothermal titration calorimetry; bp, base pair.

■ REFERENCES

(1) Neidle, S. (2001) DNA minor-groove recognition by small molecules. *Nat. Prod. Rep. 18*, 291−309.

(2) Dervan, P. B. (2001) Molecular recognition of DNA by small molecules. *Bioorg. Med. Chem. 9*, 2215−2235.

(3) Wemmer, D. E. (2000) Designed sequence-specific minor groove ligands. *Annu. Rev. Biophys. Biomol. Struct. 29*, 439−461.

(4) Barrett, M. P., Boykin, D. W., Brun, R., and Tidwell, R. R. (2007) Human African trypanosomiasis: Pharmacological re-engagement with a neglected disease. *Br. J. Pharmacol. 152*, 1155−1171.

(5) Stadler, A. L., Renikuntla, B. R., Yaron, D., Fang, A. S., and Armitage, B. A. (2011) Substituent effects on the assembly of helical cyanine dye aggregates in the minor groove of a DNA template. *Langmuir 27*, 1472−1479.

(6) Hampshire, A. J., and Fox, K. R. (2008) The effects of local DNA sequence on the interaction of ligands with their preferred binding sites. *Biochimie 90*, 988−998.

(7) Wilson, W. D., Tanious, F. A., Mathis, A., Tevis, D., Hall, J. E., and Boykin, D. W. (2008) Antiparasitic compounds that target DNA. *Biochimie 90*, 999−1014.

(8) Bailly, C., and Chaires, J. B. (1998) Sequence-specific DNA minor groove binders. Design and synthesis of netropsin and distamycin analogues. *Bioconjugate Chem. 9*, 513−538.

(9) Antonow, D., Kaliszczak, M., Kang, G. D., Coffils, M., Tiberghien, A. C., Cooper, N., Barata, T., Heidelberger, S., James, C. H., Zloh, M., Jenkins, T. C., Reszka, A. P., Neidle, S., Guichard, S. M., Jodrell, D. I., Hartley, J. A., Howard, P. W., and Thurston, D. E. (2010) Structure-Activity Relationships of Monomeric C2-Aryl Pyrrolo 2,1-c 1,4 benzodiazepine (PBD) Antitumor Agents. *J. Med. Chem. 53*, 2927−2941.

(10) Zewail-Foote, M., and Hurley, L. H. (1999) Ecteinascidin 743: A minor groove alkylator that bends DNA toward the major groove. *J. Med. Chem. 42*, 2493−2497.

(11) Buchmueller, K. L., Staples, A. M., Howard, C. M., Horick, S. M., Uthe, P. B., Le, N. M., Cox, K. K., Nguyen, B., Pacheco, K. A. O., Wilson, W. D., and Lee, M. (2005) Extending the language of DNA molecular recognition by polyamides: Unexpected influence of imidazole and pyrrole arrangment on binding affinity and specificity. *J. Am. Chem. Soc. 127*, 742−750.

(12) Nelson, S. M., Ferguson, L. R., and Denny, W. A. (2007) Non-covalent ligand/DNA interactions: Minor groove binding agents. *Mutat. Res. 623*, 24−40.

(13) Wang, L., Bailly, C., Kumar, A., Ding, D., Bajic, M., Boykin, D. W., and Wilson, W. D. (2000) Specific molecular recognition of mixed nucleic acid sequences: An aromatic dication that binds in the DNA minor groove as a dimer. *Proc. Natl. Acad. Sci. U.S.A. 97*, 12−16.

(14) Zihlif, M., Catchpoole, D. R., Stewart, B. W., and Wakelin, L. P. (2010) Effects of DNA minor groove binding agents on global gene expression. *Cancer Genomics Proteomics 7*, 323−330.

(15) Goodwin, K. D., Lewis, M. A., Long, E. C., and Georgiadis, M. M. (2008) Crystal structure of DNA-bound Co(III)-bleomycin B-2: Insights on intercalation and minor groove binding. *Proc. Natl. Acad. Sci. U.S.A. 105*, 5052−5056.

(16) Goodwin, K.D., Lewis, M. A., Tanious, F. A., Tidwell, R. R., Wilson, W. D., Georgiadis, M. M., and Long, E. C. (2006) A high-throughput, high-resolution strategy for the study of site-selective DNA binding agents: Analysis of a "highly twisted" benzimidazole-diamidine. *J. Am. Chem. Soc. 128*, 7846−7854.

(17) Tanious, F. A., Laine, W., Peixoto, P., Bailly, C., Goodwin, K. D., Lewis, M. A., Long, E. C., Georgiadis, M. M., Tidwell, R. R., and Wilson, W. D. (2007) Unusually strong binding to the DNA minor groove by a highly twisted benzimidazole diphenylether: Induced fit and bound water. *Biochemistry 46*, 6944−6956.

(18) Goodwin, K. D., Long, E. C., and Georgiadis, M. M. (2005) A host-guest approach for determining drug-DNA interactions: An example using netropsin. *Nucleic Acids Res. 33*, 4106−4116.

(19) Glass, L. S., Nguyen, B., Goodwin, K. D., Dardonville, C., Wilson, W. D., Long, E. C., and Georgiadis, M. M. (2009) Crystal structure of a trypanocidal 4,4′-bis(imidazolinylamino)diphenylamine bound to DNA. *Biochemistry 48*, 5943−5952.

(20) Chenoweth, D. M., and Dervan, P. B. (2010) Structural basis for cyclic Py-Im polyamide allosteric inhibition of nuclear receptor binding. *J. Am. Chem. Soc. 132*, 14521−14529.

(21) Chenoweth, D. M., and Dervan, P. B. (2009) Allosteric modulation of DNA by small molecules. *Proc. Natl. Acad. Sci. U.S.A. 106*, 13175−13179.

(22) Nguyen, B., Neidle, S., and Wilson, W. D. (2009) A role for water molecules in DNA-ligand minor groove recognition. *Acc. Chem. Res. 42*, 11−21.

(23) Zinzalla, G., Haque, M. R., Basu, B. P., Anderson, J., Kaye, S. L., Haider, S., Hasan, F., Antonow, D., Essex, S., Rahman, K. M., Palmer, J., Morgenstern, D., Wilderspin, A. F., Neidle, S., and Thurston, D. E. (2010) A novel small-molecule inhibitor of IL-6 signalling. *Bioorg. Med. Chem. Lett. 20*, 7029−7032.

(24) Hartley, J. A., Hamaguchi, A., Coffils, M., Martin, C. R. H., Suggitt, M., Chen, Z. Z., Gregson, S. J., Masterson, L. A., Tiberghien, A. C., Hartley, J. M., Pepper, C., Lin, T. T., Fegan, C., Thurston, D. E., and Howard, P. W. (2010) SG2285, a novel C2-aryl-substituted pyrrolobenzodiazepine dimer prodrug that cross-links DNA and exerts highly potent antitumor activity. *Cancer Res. 70*, 6849−6858.

(25) Paine, M. F., Wang, M. Z., Generaux, C. N., Boykin, D. W., Wilson, W. D., De Koning, H. P., Olson, C. A., Pohlig, G., Burri, C., Brun, R., Murilla, G. A., Thuita, J. K., Barrett, M. P., and Tidwell, R. R. (2010) Diamidines for human African trypanosomiasis. *Curr. Opin. Invest. Drugs 11*, 876−883.

(26) Anthony, N.G., Breerl, D., Clarke, J., Donoghue, G., Drummond, A. J., Ellis, E. M., Gemmell, C. G., Helesbeux, J. J., Hunter, I. S., Khalaft, A. I., Mackay, S. P., Parkinson, J. A., Suckling, C. J., and Waiah, R. D. (2007) Antimicrobial lexitropsins containing amide, amidine, and alkene linking groups. *J. Med. Chem. 50*, 6116−6125.

(27) Hadjivassileva, T., Thurston, D. E., and Taylor, P. W. (2005) Pyrrolobenzodiazepine dimers: Novel sequence-selective, DNA-interactive, cross-linking agents with activity against Gram-positive bacteria. *J. Antimicrob. Chemother. 56*, 513−518.

(28) Bando, T., Narita, A., Saito, I., and Sugiyama, H. (2002) Molecular design of a pyrrole-imidazole hairpin polyamides for effective DNA alkylation. *Chem.—Eur. J. 8*, 4781−4790.

(29) Murty, M., and Sugiyama, H. (2004) Biology of N-methylpyrrole-N-methylimidazole hairpin polyamide. *Biol. Pharm. Bull. 27*, 468−474.

(30) Crowley, K. S., Phillion, D. P., Woodard, S. S., Schweitzer, B. A., Singh, M., Shabany, H., Burnette, B., Hippenmeyer, P., Heitmeier, M., and Bashkin, J. K. (2003) Controlling the intracellular localization of fluorescent polyamide analogues in cultured cells. *Bioorg. Med. Chem. Lett. 13*, 1565−1570.

(31) Dudouet, B., Burnett, R., Dickinson, L. A., Wood, M. R., Melander, C., Belitsky, J. M., Edelson, B., Wurtz, N., Briehn, C., Dervan, P. B., and Gottesfeld, J. M. (2003) Accessibility of nuclear chromatin by DNA binding polyamides. *Chem. Biol. 10*, 859−867.

(32) Henry, J. A., Le, N. M., Nguyen, B., Howard, C. M., Bailey, S. L., Horick, S. M., Buchmueller, K. L., Kotecha, M., Hochhauser, D., Hartley, J. A., Wilson, W. D., and Lee, M. (2004) Targeting the inverted CCAAT box 2 in the topoisomerase II α promoter by JH-37, an imidazole-pyrrole polyamide hairpin: Design, synthesis, molecular biology, and biophysical studies. *Biochemistry 43*, 12249−12257.

(33) Fagan, P., and Wemmer, D. E. (1992) Cooperative binding of distamcyin A to DNA in the 2:1 mode. *J. Am. Chem. Soc. 114*, 1080−1081.

(34) Pelton, J. G., and Wemmer, D. E. (1989) Structual characterization of a 2-1 distamcyin A-d(CGCAAATTGGC) complex by two-dimensional NMR. *Proc. Natl. Acad. Sci. U.S.A. 86*, 5723−5727.

(35) Pelton, J. G., and Wemmer, D. E. (1988) Structural modeling of the distamycin A-d$(CGCGAATTCGCG)_2$ complex using 2D NMR and molecular mechanics. *Biochemistry 27*, 8088−8096.

(36) Coll, M., Aymami, J., Vandermarel, G. A., Vanboom, J. H., Rich, A., and Wang, A. H. J. (1989) Molecular-structure of the netropsin-d(CGCGATATCGCG) complex: DNA conformation in an alternating AT segment. *Biochemistry 28*, 310−320.

(37) Pelton, J. G., and Wemmer, D. E. (1990) Binding modes of distamcyin A with d$(CGCAAATTTGCG)_2$ determined by 2-dimensional NMR. *J. Am. Chem. Soc. 112*, 1393−1399.

(38) Tevis, D. S., Kumar, A., Stephens, C. E., Boykin, D. W., and Wilson, W. D. (2009) Large, sequence-dependent effects on DNA conformation by minor groove binding compounds. *Nucleic Acids Res. 37*, 5550−5558.

(39) Hunt, R. A., Munde, M., Kumar, A., Ismail, M. A., Farahat, A. A., Arafa, R. K., Say, M., Batista-Parra, A., Tevis, D., Boykin, D. W., and Wilson, W. D. (2011) Induced changes in DNA complexes: Influence of DNA sequences and small molecule structures. *Nucleic Acids Res. 39*, 4265−4274.

(40) Wemmer, D. E., Geierstanger, B. H., Fagan, P. A., Dwyer, T. J., Jacobsen, J. P., Pelton, J. G., Ball, G. E., Leheny, A. R., Chang, W.-H., Bathini, Y., Lown, J. W., Rentzeperis, D., Marky, L. A., Singh, S., and Kollman, P. (1994) Minor groove recognition of DNA by distamycin and its analogs. In *Structural Biology: The state of the art* (Sarma, R. H., and Sarma, M. H., Eds.) pp 301−323, Adenine Press, New York.

(41) Bailly, C., Minnock, A., and Waring, M. J. (1996) A simple ligation assay to detect effects of drugs on the curvature flexibility of DNA. *FEBS Lett. 396*, 253−256.

(42) Crothers, D. M., and Drak, J. (1992) Global features of DNA-structure by comparative gel-electrophoresis. *Methods Enzymol. 212*, 46−71.

(43) Fasman, G. D. (1975) *Handbook of Biochemistry and Molecular Biology*, 3rd ed., pp 589, CRC Press, Boca Raton, FL.

(44) Ross, E. D., Den, R. B., Hardwidge, P. R., and Maher, L. J. (1999) Improved quantitation of DNA curvature using ligation ladders. *Nucleic Acids Res. 27*, 4135−4142.

(45) Liu, Y., and Wilson, W. D. (2010) Quantitative analysis of small molecule-nucleic acid interactions with a biosensor surface and surface plasmon resonance detection. *Methods Mol. Biol. 613*, 1−23.

(46) Nanjunda, R., Munde, M., Liu, Y., and Wilson, W. D. (2011) Real-time monitoring of nucleic acid interactions with Biosensor

Plasmon Resonance. *Methods for Studying DNA/Drug Interactions* (in press).

(47) Nguyen, B., Tanious, F. A., and Wilson, W. D. (2007) Biosensor-surface plasmon resonance: Quantitative analysis of small molecule-nucleic acid interactions. *Methods 42*, 150−161.

(48) Hofstadler, S. A., and Griffey, R. H. (2001) Analysis of noncovalent complexes of DNA and RNA by mass spectrometry. *Chem. Rev. 101*, 377−390.

(49) Rosu, F., De Pauw, E., and Gabelica, V. (2008) Electrospray mass spectrometry to study drug-nucleic acids interactions. *Biochimie 90*, 1074−1087.

(50) Liu, Y., Kumar, A., Depauw, S., Nhili, R., David-Cordonnier, M.-H., Lee, M. P., Ismail, M. A., Farahat, A. A., Say, M., Chackal-Catoen, S., Batista-Parra, A., Neidle, S., Boykin, D. W., and Wilson, W. D. (2011) Water-mediated binding of agents that target the DNA minor groove. *J. Am. Chem. Soc. 133*, 10171−10183.

(51) Munde, M., Kumar, A., Nhili, R., Depauw, S., David-Cordonnier, M. H., Ismail, M. A., Stephens, C. E., Farahat, A. A., Batista-Parra, A., Boykin, D. W., and Wilson, W. D. (2010) DNA minor groove induced dimerization of heterocyclic cations: Compound structure, binding affinity, and specificity for a TTAA site. *J. Mol. Biol. 402*, 847−864.

(52) Koo, H. S., Wu, H. M., and Crothers, D. M. (1986) DNA bending at adenine·thymine tracts. *Nature 320*, 501−506.